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Quantification of Urinary Catecholamines, their Abundant Metabolites, and 5-Hydroxyindoleacetic Acid by High Performance Liquid Chromatography and Electrochemical Detection, Using a Single Mobile Phase and Uniform Isocratic Conditions

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Summary: A simple and flexible isocratic HPLC procedure was developed for the measurement of catecholamines and their abundant metabolites (e. g. normetanephrine, metanephrine, 3-methoxy-tyramine, vanillylmandelic acid, homovanillic acid) and 5-hydroxyindoleacetic acid by ion-pair reversed-phase chromatography on C18 columns, using a single mobile phase containing both sodium octane sulphonate and diethylamine as ion-pairing reagents.

Introduction

Differential diagnosis of phaeochromatocytoma (1–3), neuroblastoma (4, 5) and related diseases (6) requires multiple investigations, among which the determination of the catecholamines and their metabolites is of primary importance. Comprehensive biochemical data enable the clinician to reach an early decision and to initiate an appropriate therapy.

Nowadays, the catecholamines and each group of their metabolites are generally determined by HPLC using different sample preparation procedures and different mobile phases and separating conditions. *Parker* et al. (6) described a method for quantifying urinary catecholamines and some of their metabolites under uniform HPLC conditions. In this paper we present a newly developed method using a single mobile phase for an easy-to-perform determination of urinary epinephrine, norepinephrine, dopamine, normetanephrine, metanephrine, 3-methoxytyramine, vanillylmandelic acid, homovanillic acid, and 5-hydroxy-indoleacetic acid.

Materials and Methods

Apparatus and evaluation

The HPLC system used consisted of a Model 2249 pump (Pharmacia-LKB, Freiburg, Germany), a Model 2157 autosampler (Pharmacia-LKB, Freiburg, Germany), a column oven (5–85 °C) and a Model 460 electrochemical detector (Waters, Eschborn, Germany). The pump and sampler were PC-controlled by an HPLC-Manager® from Pharmacia-LKB. Concentrations of detected compounds were also PC-calculated, using integration software, 2600 Version 5.0 (Nelson, Pharmacia-LKB, Freiburg, Germany) which measures the areas under the detected peaks, relates them to external standards and multiplies them with the recovery factor of an internal standard. The statistical data were processed by Lotus 1-2-3 for Windows®.

Mobile phase and column

The mobile phase consisted of 4.7 g monochloroacetic acid, 1.8 g NaOH, 0.51 g Na_2EDTA, 0.15 g sodium octane sulphonate, 10.5 g citric acid monohydrate and 450 µl diethylamine made up to one litre with a water/acetonitrile mixture (92 + 8 or 96 + 4, depending on the column size used). The pH of the solutions was adjusted to 3.0 with monochloroacetic acid or NaOH.

Two types of C18 columns were tested: a Waters 5 µm, 3.9 × 300 mm, C18 Nova Pak (A), and a Pharmacia 3 µm, 4 x125 mm, SuperPac Spherisorb® ODS2 (B). Most of the analyses were done with column A, using the 92 + 8 water/acetonitrile mixture as the mobile phase. Column B was used with the 96 + 4 water/acetonitrile mixture.

Chromatographic conditions

Because of the high oxidation potential of isovanillylmandelic acid, which is used as an internal standard for the vanillylmandelic acid-group, the potential used for detecting all the investigated compounds was between 0.75 V and 0.8 V with a Ag/AgCl reference electrode. The mobile phase flow rate was 0.8 ml/min, and the column temperature was held at 25 °C.

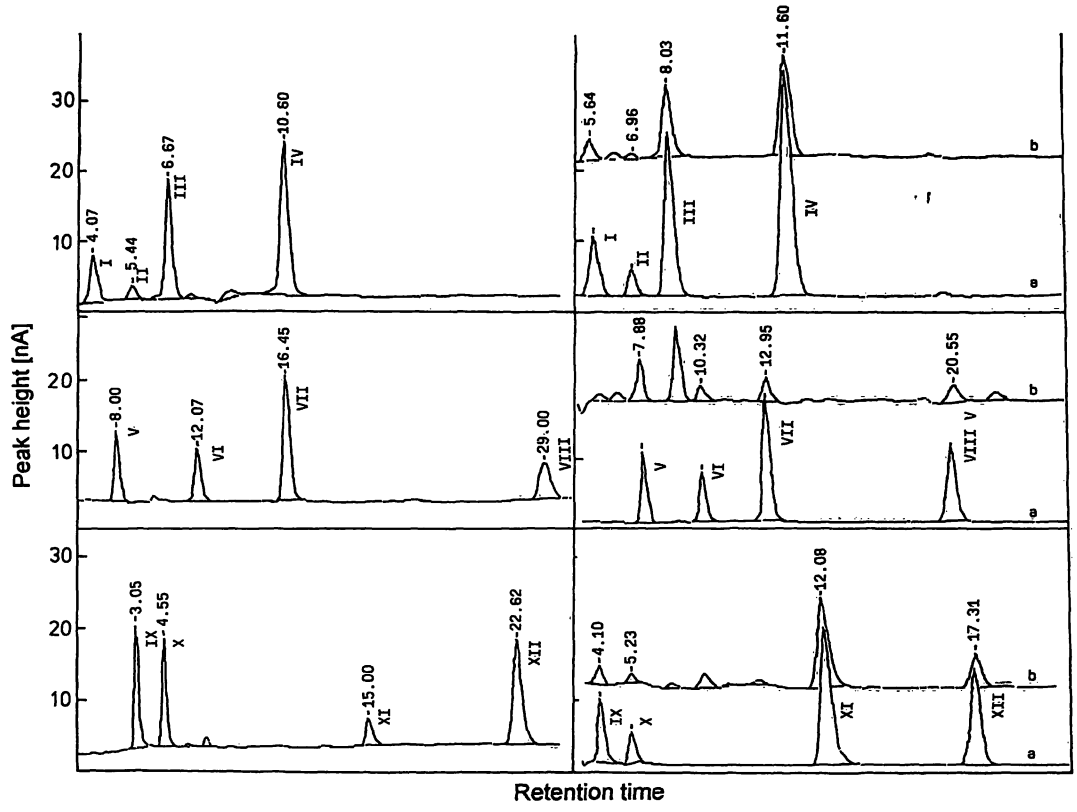

Fig. 1 Typical chromatograms of norepinephrine = I, epinephrine = II, dopamine = IV (top), and normetanephrine = V, metanephrine = VI and 3-methoxytyramine = VIII (middle); and vanillylmandelic acid = IX, 5-hydroxy-indoleacetic acid = XI, and homovanillic acid = XII (bottom) under the chromatographic conditions stated in the text:
(right) chromatograms of standards (a) and urine patient samples (b), column A (Waters, C18 Nova Pak® with 5 µm, 3.9 × 300 mm size);
(left) chromatograms of standards, column B (Pharmacia, SuperPac Spherisorb® ODS2 with 3 µm, 4 × 125 mm size);
the numbers beside the peaks are the retention times;
III, VII and X are the internal standards of dihydroxybenzylamine, 3-methoxy-4-hydroxy-benzylamine, and isovanillylmandelic acid.

Procedure

Specimen collection

After centrifugation of an aliquot of 24-h urine, acidified with 10 ml of concentrated HCl, the supernatant was stored at −20 °C until processing.

Preparation

Extraction of epinephrine, norepinephrine and dopamine

Free catecholamines are extracted from urine using a cation-exchange column (Pharmacia-LKB, Freiburg, Germany). To 3 ml of filtered urine, buffered with 6 ml of Na_2EDTA (1 g/l H_2O), 100 µl of internal standard (dihydroxybenzylamine, 1.5 mg/l 0.2 mol/l HCl) were added. This mixture was adjusted to pH 3.0−7.0 with NaOH and applied to the column. The column was washed once with 10 ml ammonia (0.05 mol/l), and twice with one column filling of water, and the catecholamines were eluated with 6 ml of mobile phase. An aliquot (30 µl) of the eluate was injected into the HPLC system.

Extraction of metanephrine, normetanephrine and 3-methoxytyramine

This group of catecholamine metabolites was extracted from urine after acidic hydrolysis using a cation-exchange column (Pharmacia-LKB, Freiburg, Germany). To 2 ml of filtered, acidified urine were added 100 µl of internal standard (3-methoxy-4-hydroxy-benzylamine, 2 mg/l water) in a stoppered tube and the pH was adjusted to 0.8−1.0. The solution was incubated for 30 minutes at 90−100 °C to hydrolyse sulphate and glucuronide conjugates, then cooled to room temperature. The hydrolysed sample was diluted with 6 ml of buffer (Na_2 · EDTA, 1 g/l water), adjusted to pH 6.0−10.0 with NaOH, and applied to the cation-exchange column (Pharmacia-LKB, Freiburg, Germany). The column was washed in three steps, with 10 ml water, with 3 ml boric acid (40 g/l H_2O) and at least with 5 ml water. Metanephrine, normetanephrine and 3-methoxytyramine were eluted from the column with 5 ml of mobile phase solution, and 50 µl of this eluate were injected into the HPLC system.

Extraction of vanillylmandelic acid, homovanillic acid and 6-hydroxyindoleacetic acid

The acidic metabolites were extracted from urine with an anion-exchange column (Pharmacia-LKB, Freiburg, Germany). We added 40 µl of internal standard (isovanillylmandelic acid, 250 mg/l 0.2 mol/l HCl) to 1 ml of acidified urine, and diluted this mixture with 4 ml of water (HPLC quality). The pH was adjusted to 5.5−6.5 with NaOH, and 1.0 ml of the resulting solution was applied to the column. The column was washed once with 3 ml of wash buffer I (ammonia, 0.05 mol/l), and twice with wash buffer II (boric acid, 40 g/l water). Vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid were eluated with 2 ml of mobile phase solution. Ten µl of the eluate were injected into the HPLC system.

Standardization

Three stock solutions of external standards were prepared.

Stock solution 1:

2.5 µg of norepinephrine, 0.5 µg of epinephrine, 10.0 µg of dopamine and 5.0 µg of dihydroxybenzylamine (internal standard) were dissolved in 100 ml 0.2 mol/l HCl. This stock solution is stable for min. six months at 4 °C.

Stock solution 2:

5.0 µg of normetanephrine, 5.0 µg of metanephrine, 5.0 µg of 3-methoxytyramine and 10.0 µg of 3-methoxy-4-hydroxy-benzylamine (internal standard) were dissolved in 100 ml 0.2 mol/l HCl. This solution was also stable for at least six months at 4 °C.

Stock solution 3:

50 µg of vanillylmandelic acid, 50 µg of homovanillic acid, 50 µg of 5-hydroxyindoleacetic acid and 50 µg of isovanillylmandelic acid (internal standard) were dissolved in 100 ml 0.2 mol/l HCl. This solution was stable for about one week at 4 °C. Small portions were kept frozen at 20 °C until they were assayed.

The concentration of the catecholamines and their metabolites in the unknown samples were calculated by comparison of the peak areas of the substances to be determined and that of the external standard.

Results

Chromatography

Figure 1 shows representative chromatograms for each group of catecholamines and metabolites, separated on column A and column B. Two columns were used to document the applicability of the chosen mobile phase to various column types. The retention times for all compounds range between 4.0 min for vanillylmandelic acid and 21.0 min for 3-methoxytyramine. The constituents are well separated.

Imprecision

Intra- and inter-assay imprecisions as determined from analyses of Pharmacia normal and pathological control urines and from a normal urine, are summarized in tables 1 and 2. Intra-assay coefficients of variation (CV) were between 1.6% for vanillylmandelic acid and 6.5% for epinephrine in control urines. In normal urine, CVs ranged from 2.7% for vanillylmandelic acid to 11.1% for epinephrine. The inter-assay CVs in controls were between 4.1% for vanillylmandelic acid and 7.8% for epinephrine. In normal urine, the CVs ranged from 6.5% for vanillylmandelic acid to 14.5% for epinephrine.

Linearity and recovery

Known quantities of each investigated component were added to normal urines to test the linearity and the accuracy of the method. The weighed amounts, the original concentration of the components in the urine and the linear regression data are presented in table 3. The amount measured in each supplemented sample minus the value of analyte in the urine was compared with the known amount added. These data are listed in table 4.

Tab. 1 Intra-assay imprecision of the method in normal, pathological control urine and a normal urine pool.

Analyte		n	$\bar{x}$	s	CV (%)
Control urine (normal)					
Norepinephrine	nmol/l	20	387.1	10.6	2.8
Epinephrine	nmol/l	20	128.3	8.2	6.5
Dopamine	nmol/l	20	1302	22.2	1.7
Normetanephrine	nmol/l	20	1807	68.7	3.8
Metanephrine	nmol/l	20	1446	23.8	1.7
3-Methoxytyramine	nmol/l	20	998.2	29.3	2.9
Vanillylmandelic acid	µmol/l	20	28.7	0.5	1.6
5-Hydroxy-indoleacetic acid	µmol/l	20	58.3	1.2	2.0
Homovanillic acid	µmol/l	20	41.1	0.9	2.2
Control urine (pathological)					
Norepinephrine	nmol/l	20	1176	33.1	2.8
Epinephrine	nmol/l	20	233.1	9.3	4.0
Dopamine	nmol/l	20	2681	64.0	2.4
Normetanephrine	nmol/l	20	7864	333.2	4.2
Metanephrine	nmol/l	20	3095	117.1	3.8
3-Methoxytyramine	nmol/l	20	2373	97.5	4.1
Vanillylmandelic acid	µmol/l	20	84.3	2.0	2.4
5-Hydroxy-indoleacetic acid	µmol/l	20	300.6	8.4	2.8
Homovanillic acid	µmol/l	20	102.1	2.7	2.7
Patient urine					
Norepinephrine	nmol/l	20	457.4	35.5	7.8
Epinephrine	nmol/l	16	40.9	4.4	11.1
Dopamine	nmol/l	20	1144	90.8	7.9
Normatanephrine	nmol/l	19	946.0	45.8	4.8
Metanephrine	nmol/l	20	450.0	20.8	4.6
3-Methoxytyramine	nmol/l	20	347.5	12.0	3.4
Vanillylmandelic acid	µmol/l	20	10.2	0.3	2.7
5-Hydroxy-indoleacetic acid	µmol/l	20	54.7	1.5	2.8
Homovanillic acid	µmol/l	20	19.6	0.4	2.1

Detection limits

The determination of the lower detection limits of the components is based on the linear dilution of their standards.

The detection limits for norepinphrine, epinephrine and dopamine were 28.0, 21.0 and 124 nmol/l, respectively, using 30 µl injection volume of a standard dilution. For normetanephrine, metanephrine and 3-methoxytyramine the detection limits were 39.0, 36.0 and 128 nmol/l, respectively, using 50 µl injection volume of a standard solution. The lower detection limits of vanillylmandelic acid, 5-hydroxy-indoleacetic acid and homovanillic acid were 3.6, 3.7 and 3.9 µmol/l, respectively, using 10 µl injection volume.

Tab. 2 Inter-assay imprecision of the method in normal, pathological control urine and a normal urine pool.

Analyte		n	$\bar{x}$	s	CV (%)
Control urine (normal)					
Norepinephrine	nmol/l	19	426.7	24.8	5.8
Epinephrine	nmol/l	16	120.0	10.9	9.1
Dopamine	nmol/l	20	1381	75.8	5.5
Normetanephrine	nmol/l	20	1648	32.2	2.0
Metanephrine	nmol/l	20	1501	39.6	2.6
3-Methoxytyramine	nmol/l	20	1053	36.5	3.5
Vanillylmandelic acid	nmol/l	18	31.1	2.0	6.5
5-Hydroxy-indoleacetic acid	µmol/l	19	56.0	4.5	8.1
Homovanillic acid	µmol/l	19	40.5	2.2	5.4
Control urine (pathological)					
Norepinephrine	nmol/l	20	1196	68.6	5.7
Epinephrine	nmol/l	14	227.6	18.0	7.8
Dopamine	nmol/l	20	2553	173.7	6.8
Normetanephrine	nmol/l	20	9531	333.3	3.5
Metanephrine	nmol/l	20	3925	131.8	3.4
3-Methoxytyramine	nmol/l	20	2171	81.3	3.7
Vanillylmandelic acid	µmol/l	20	86.3	3.5	4.1
5-Hydroxy-indoleacetic acid	µmol/l	20	253.2	14.2	5.6
Homovanillic acid	µmol/l	20	103.2	4.9	5.0
Patient urine					
Norepinephrine	µmol/l	20	427.3	22.5	5.2
Epinephrine	µmol/l	14	30.0	4.4	14.5
Dopamine	µmol/l	20	1823	62.1	3.4
Normetanephrine	µmol/l	20	2423	63.3	2.6
Metanephrine	µmol/l	20	354.0	21.3	6.1
3-Methoxytyramine	µmol/l	18	1025	35.9	8.6
Vanillylmandelic acid	µmol/l	20	10.4	0.6	5.6
5-Hydroxy-indoleacetic acid	µmol/l	20	50.4	2.2	4.3
Homovanillic acid	µmol/l	20	19.5	0.5	2.6

Tab. 4 Analytical recovery and accuracy.

Analyte	Weighed amount added to urine [µmol/l]	Recovered [µmol/l]	Recovery [%]
Norepinephrine	5.7	6.1	107
	1.7	1.9	113
	0.24	0.26	111
Epinephrine	4.4	4.31	98
	2.2	2.12	96
	0.44	0.38	89
Dopamine	5.3	5.9	111
	2.65	2.95	111
	1.85	1.98	107
Normetanephrine	10.9	11.07	102
	5.45	6.44	118
	0.55	0.54	99
Metanephrine	7.6	8.1	107
	5.1	5.6	112
	1.3	1.4	108
3-Methoxytyramine	9.0	9.5	106
	6.0	6.5	109
	1.5	1.5	100
Vanillylmandelic acid	459	512	103
	306	335	110
	92	103	112
5-Hydroxy-indoleacetic acid	407	411	99
	110	110	100
	39	41	94
Homovanillic acid	330	384	107
	132	136	103
	83	84	101

Tab. 3 The linearity of the method and recovery.

Analyte	Linear regression equations	r	Concentration in native urine [µmol/l]	Weighed amount added to urine [µmol/l]	Total content [µmol/l]	Detected content [µmol/l]	Recovery [%]
Norepinephrine	$y = 1.43x + 0.21$	0.999	0.40	23.62	24.02	23.64	98.4
Epinephrine	$y = 1.06x - 0.01$	0.999	0.15	22.00	22.15	21.83	98.6
Dopamine	$y = 1.19x + 1.03$	0.999	1.11	26.49	27.60	26.13	94.7
Normetanephrine	$y = 0.99x + 1.04$	0.990	0.71	13.63	14.34	13.65	95.2
Metanephrine	$y = 1.16x + 0.50$	0.995	0.49	12.56	13.05	12.68	97.2
3-Methoxytyramine	$y = 0.91x + 0.57$	0.992	0.34	15.03	15.37	14.95	97.3
Vanillylmandelic aicd	$y = 1.15x + 7.47$	0.998	12.6	609	621.6	605	97.3
5-Hydroxy-indoleacetic acid	$y = 1.72x - 43.83$	0.936	24.8	548	572.8	633	110.5
Homovanillic acid	$y = 1.45x - 12.83$	0.989	15.4	660	675.4	659	97.6

Discussion

The catecholamines and their methoxy derivatives, metanephrine, normetanephrine and 3-methoxy-tyramine, are basic compounds, whereas vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid are acidic components. Thus, they can be separated by cation- and anion-exchange chromatography, respectively, or by ion-pair reversed-phase chromatography using different mobile phase. Because of the diversity of catecholamine-producing tumours, a complete analysis of the catecholamines and their methoxy and acidic

metabolites is clinically indicated in many cases. Uniform analytical conditions contribute to a better practicability of the method in the clinical chemical laboratory and to a reduction of analysis time and costs. As an approach to this analytical goal we suggest a single mobile phase for the HPLC separation of all three groups of components. We used a mixture of sodium octane sulphonate and diethylamine in the mobile phase in order to form ion-pairs for both amines and acidic compounds. Peak discrimination is also enhanced by optimization of the pH of the mobile phase. Good results are obtained with pH 3.0 (7, 8). Uniformity is also achieved in the sample preparation procedure by using the mobile phase to elute all three classes of analytes from the solid-phase extraction cartridges. Using mobile phase in the sample work-up procedure also reduces interferences in the analysis.

The mobile phase can be recycled and one column has been used for more than 2000 analyses. In contrast to the method described by *Parker* et al. (6), our method also determines 3-methoxy-tyramine and homovanillic acid. In addition, we were able vary the size of the column according to the ratio of acetonitrile/water used in the mobile phase.

The method described is simple, practicable and reliable, and suitable for routine determinations of the catecholamines and all of their clinically relevant metabolites in the diagnosis of catecholamine-producing tumours.

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